BACKGROUND OF THE INVENTION

1. Field of Invention

The present invention generally relates to the enhancement of immunohistochemical staining of fixed tissue samples. More particularly, an embodiment of the invention relates to the enhancement of immunohistochemical staining of embedded formalin-fixed tissue samples using a single composition in a single step.

2. Description of Related Art

Tissue sections obtained from clinical or animal experimentation frequently have been fixed, embedded and stored in a form suitable for later examination by microscopy. Traditional fixation methods frequently have employed aldehyde fixatives, which fix the tissue by causing cross-linking reactions within and between tissue proteins. Cross-links tend to preserve tissue morphology and integrity, harden the tissue for slicing, and inhibit microbial attack. After tissue samples have been fixed, they are typically embedded in an embedding medium so that the samples may be cut into thin sections. Paraffin is the most common embedding medium, although acrylamide and celloidin may also be used.

Aldehyde fixation tends to cause substantial changes to the structure of the [issue] tissue sample. These changes often tend to cause the antigens that may be present in the tissue samples to lose their reactivity toward antibodies that target such antigens. One effect of formalin fixation is to substantially lock the three dimensional shape of protein molecules within the tissue samples. Because of the recent development of new immunohistochemical reagents, immunohistochemical analyses may now be performed that were impossible to perform at the time many tissues were

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originally stored. Therefore, a number of procedures have been developed which could reverse some of the changes produced by aldehyde fixation, and enhance the immunohistochemical staining properties of the tissue sample.

One method for improving the staining abilities of tissue samples which have been fixed in formalin and embedded in acrylamide gel relates to treatment of acrylamide gel embedded tissue in 1.0% 2-mercaptoethanol for 15 minutes, followed by rinsing with phosphate buffered saline. This treatment allowed the tissue samples to be stained by a number of staining reagents. A method for restoring the immunohistochemical staining properties of tissue samples is described in U.S. Patent No. 5,244,787 to Key et al. This method involved removing the embedding medium in a pretreatment step. For Paraffin-embedded tissue samples, this pretreatment was accomplished by clearing the tissue samples in xylenes and rehydrating the samples. After the embedding medium has been removed, the sample may be heated in either de-ionized water, an aqueous solution of a zinc salt, or an aqueous solution of a lead salt. The tissue samples were reported to show improved immunohistochemical staining properties when heated in a microwave oven. Improvement was reportedly seen when the solution was heated to its boiling temperature. In general, microwave heating appears to have been found by Key et al to give better results than conventional heating. Solutions containing zinc or lead salts apparently gave significantly better results than de-ionized water.

Another method for restoring the immunohistochemical staining properties of tissue samples is described in U.S. Patent No. 5,578,452 by Shi et al. In this method the formalin-fixed embedded tissues were treated with a solution of an aldehyde releasing reagent. The aldehyde releasing reagent

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may release aldehyde from the tissue sample by reacting with the aldehyde in a substantially irreversible manner to form a non-aldehyde derivative.

A number of investigators have investigated the importance of several reaction conditions with respect to enhancement of immunohistochemical staining ability. In general it has been found that the pH of the solution and temperature tend to have the most effect on the staining ability of the tissue samples. In general, with some limitations, the higher the temperature during the enhancement of the tissue samples, the better the staining enhancement tends to be. The effect of pH tends to be dependent on the type of antibody being used in the staining process and may be optimized for the antibodies to be used during the staining procedure.

The above mentioned methods inadequately address, among other things, the restoration of paraffin embedded tissue samples in a single reaction step. The procedures described above are usually performed on deparaffinized samples. Typically, the paraffin embedding medium is removed from the samples by successive immersion through a series of xylenes. Following removal of the paraffin embedding medium, the tissue must then be rehydrated by treatment with a series of ethanol-water solutions ranging typically from 100% ethanol to 90% ethanol. Finally, after the sample is rehydrated, the sample must be treated with a solution to reverse the effects of formalin fixation (also known as unmasking. It is therefore desirable that a single solution be provided derived that allows the steps of deparaffination (or de-embedding), rehydration, and unmasking of embedded tissue samples to be combined.

a step 2

SUMMARY OF THE INVENTION

An embodiment of the invention relates to a liquid composition for enhancing the immunohistochemical staining ability of tissue samples. The composition preferably includes an

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aqueous solution of a removing agent and a tissue activating agent. The composition preferably substantially simultaneously: (i) removes the embedding medium from the tissue; (ii) improves immunohistochemical staining of the tissue in comparison to tissue that has not been contacted with the composition; and (iii) substantially hydrates the tissue. The removing agent is adapted to substantially remove an embedding medium from a tissue sample. The removing agent is preferably an emulsifier, more preferably a surfactant. The removing agent preferably includes one or more of an amphoteric, anionic, cationic, or nonionic surfactant. The tissue activating agent is adapted to alter the morphology of a component of the tissue sample. The tissue activating agent preferably includes a buffering agent or a metal salt. The pH of the composition is preferably adjusted to lie between 5 to 10 by addition of an acid or base.

In another embodiment the composition preferably includes an aqueous solution of SIMPLE GREEN. SIMPLE GREEN is a non-toxic, biodegradable, environmentally safe detergent concentrate which may provide a mixture of emulsifiers. The composition may include a buffering agent. The pH of the solution is preferably adjusted to lie between 5 and 10 by addition of an acid or a base. The composition preferably substantially simultaneously: (i) removes the embedding medium from the tissue; (ii) improves immunohistochemical staining of the tissue in comparison to tissue that has not been contacted with the composition; and (iii) substantially hydrates the tissue.

In another embodiment of the composition the removing agent includes aqueous solutions of at least one of the following emulsifiers, including detergents and surfacants: Igepal-630 (sigma #3021); Tween 20 (sigma #P7949); Brij 35 (sigma #P1254); Brij 90 (sigma #P1254); Triton X-100 (sigma #T9284); CD TAB (sigma #C5335); and Tween 80 (sigma #P8074). Reference to "sigma"

surfactants

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An aqueous solution composition for enhancing antigenicity comprises up to about 25% by volume of surfactants. is provide

is reference to © 1999 Sigma-Aldrich catalog entitled: "Biochemicals and Reagents for Life Science Research", which is incorporated herein by reference.

The composition is preferably used to enhance the immunohistochemical staining of the tissue sample. In a preferred method the tissue samples are cut into sections of less than 5 microns. The tissue samples may be mounted on a positively charged slide. The sections are preferably dried at about 58°C for one hour. After this time the samples are preferably submersed within the composition. The tissue samples are preferably heated to a temperature of about 120°C at a pressure of about 10 p.s.i. for about 10-15 minutes. After heating the tissue samples are preferably placed in a container containing unused composition at a temperature of at least 90°C for up to about 5 minutes. The tissue sample may be washed with an aqueous solution of a buffering agent before staining. An advantage of the present invention relates to the use of a single composition, in a single step, to enhance the immunohistochemical staining ability of tissue samples.

Another advantage of the present invention is that the sample may be used on Paraffin-embedded tissues without removing the paraffin embedding medium prior to treatment. Yet another advantage of the present invention is to provide a non-toxic, biodegradable composition for pretreatment of slides. Yet another advantage of the present invention is that the composition preferably substantially simultaneously: (i) removes the embedding medium from the tissue; (ii) improves immunohistochemical staining of the tissue in comparison to tissue that has not been contacted with the composition; and (iii) substantially hydrates the tissue.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Tissue sections obtained from clinical or animal experimentation frequently are fixed, embedded and stored in a form suitable for later examination by light microscopy. Traditional fixation methods frequently have employed aldehyde fixatives, especially formaldehyde, which preserves the integrity of the tissue samples as well as protect the sample from microbial attack. One of the major features of this fixation process is that the tissue antigens tend to be masked, that is, the antigens within the tissue are no longer reactive toward antibodies. When aldehyde based fixatives are used, this masking of the antigens is thought to be due to the reaction of the aldehyde with the tissue proteins. During the fixation process, the aldehyde presumably fixes the tissue by causing cross-linking reactions within and between tissue proteins, as well as causing other unknown changes to the tissue structure. These cross-links within the tissue proteins tend to alter the three dimensional shape of the protein, preventing access of antibodies to the antigens. After tissue samples have been fixed, they are typically embedded in an embedding medium, such as paraffin or celloidin, so that the samples may be cut into thin sections. The embedding process is preferably accomplished by soaking the tissue samples within the embedding medium such that the tissue samples are substantially surrounded by the embedding medium. In many cases the embedding medium may also soak into the interior of the tissue samples. The embedding medium may prevent the tissue samples from being stained during an immunohistochemical staining procedure.

Recently, new techniques in immunohistochemical staining of tissue samples have been developed. In general tissue samples are studied for the presence of different types of cells. The specific type of cells being studied may be stained, in the presence of other cells, by the application of immunohistochemical staining techniques. During an immunohistochemical staining process the

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tissue sample may be reacted with an antibody which specifically binds with the type of cells being studied, and no other type of cells. The bound cell-antibody complex may now be stained, without staining any of the other cells, to allow the stained cells to be easily distinguished from the other cells in the tissue sample. These techniques typically require the antigens to be unmasked before use. Because of the wide spread use of formaldehyde as a fixation chemical or a constituent thereof, it is desirable to derive a procedure whereby the antigenic activity of these tissue samples may be restored. Such a procedure may also take into account the embedding medium of the tissue samples. Formalin-fixed tissue samples are commonly embedded in paraffin before use. Removal of a portion of the embedding medium is thought to be necessary before immunohistochemical staining may be accomplished.

The enhancement of immunohistochemical staining of embedded, fixed tissue samples (typically formalin-fixed tissue samples) may be achieved by treating the tissue samples with an appropriate liquid composition. The composition preferably improves the staining of the tissue samples by accomplishing three effects. These effects may be accomplished substantially simultaneously. First, the composition may substantially remove the embedding medium from the tissue sample. Removal of the embedding medium allows the penetration of the immunohistochemical stains into at least a portion of the tissue samples preserved by the fixation process. Finally, the composition preferably rehydrates the tissue sample. During the process of fixation most of the water is removed from the tissue sample, rehydration of the tissue sample may allow the tissue to change its morphological structure and/or attain its original morphological structure.

ionic. The removing agent may be taken from any of these classes of surfactants. Preferably, the removing agent may be comprised of a mixture of compatible surfactants taken from one or more of these classes.

Nonionic surfactants include molecules that contain a substantially polar functional group attached to a substantially non-polar group.

The tissue activating agent is preferably adapted to interact with the tissue sample such that the morphology of the components of the tissue are altered. In general the tissue sample contains a number of biological components, including proteins, and nucleic acids. Each of these components have a specific three dimensional structure related to the composition of the component. During the fixation, the tissue sample may be treated with an aqueous solution of formaldehyde. The formaldehyde reacts with the components to alter the three dimensional shape of these components, i.e. alter the morphology. These alterations tend to make the tissue samples substantially unreactive toward various immunohistochemical staining protocols. The tissue activating agent is preferably adapted to further alter the morphology of the tissue samples, such that the tissue samples are more reactive toward immunohistochemical reagents. While the tissue activating agent restores some of the reactivity of the tissue samples, it may not be necessary that the tissue be restored to its original morphology to increase the reactivity of the samples toward immunohistochemical stains.

A number of metal salts may be used as a tissue activating agent. Salts useful in the composition include, but are not limited to: aluminum chloride, sodium chloride, sodium fluoride, iron chloride, zinc sulfate, and lead thiocyanate. In general aqueous solutions containing these and other metal salts improve the immunohistochemical staining ability of tissue samples to a greater extent than de-ionized water. It is believed that these salts may exert an effect on the morphology,

detachment of the tissue sample from the slide. After the samples have been mounted they may be dried for at least one hour at a temperature of about ~~[58°C]~~ 58°C.

The mounted samples may then be contacted with a composition, prepared according to the previously described embodiments, and heated to a temperature of at least about 80°C for a time period of at least about 10 minutes. The samples may be contacted with the composition by submersing a portion of the tissue sample within the composition. The samples are preferably substantially submerged within the composition. Heating may be accomplished by a number of heating devices including, but not limited to: autoclaves, pressure cookers, water baths, microwave ovens, and steam heating. In general it is preferred that the slides are heated to a temperature of at least 80°C for a time period of at least 50 minutes; more preferably the slides are heated at least about 100°C for a time period of at least about 30 minutes; more preferably still the slides are heated at a temperature of at least about 110°C for a period of at least about 20 minutes. The slides may be heated at atmospheric pressure. When a pressure cooker is used as the heating source the slides are preferably heated to a temperature up to about 120°C and a pressure of up to about 2 atmospheres.

The heating is preferably sustained for a time such that the samples also become substantially hydrated. During the embedding process the water in the tissue samples is typically removed by washing the tissue samples in an alcohol. To perform an immunohistochemical staining procedure upon the tissue samples it is preferred that the tissue is substantially saturated in water. These hydrated tissue samples typically exhibit improved immunohistochemical staining over unhydrated tissue samples.

After the slides have been heated in the composition they may be washed with an appropriate buffer solution to remove composition remaining on the slide. Alternatively, after the slides have

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been heated in the composition, they may be removed and washed with a second composition prepared according to the previously described embodiments. The second composition may include the same components as the initial composition. The second composition may be at least at room temperature. Preferably, the second composition is heated to a temperature of at least about 90°C before the slides are contacted with the second composition. After contacting the slides with the second composition for about 5 minutes the slides may be additionally washed with an appropriate buffer solution to remove any of the composition remaining on the slide. The tissue samples may be stained at this point according to standard immunohistochemical staining protocols.

Examples of the composition and method will now be described in more detail. These examples are merely illustrative of the composition and method of the invention and are not intended to be limiting.

EXAMPLES

Exp. #1 Preparation of the Tissue Enhancing Composition.

A buffered solution was prepared by dissolving the trisodium citrate dihydrate (2.9 g, 0.01 moles) in de-ionized water (1000 mL). Approximately 9 drops of concentrated hydrochloric acid were added to the solution. The pH of the solution was then determined. If the pH value was greater than 6.04, additional drops of concentrated hydrochloric acid were added until the pH dropped below 6.04. If the pH was below 5.96, drops of a 4N potassium hydroxide solution were added until the pH was greater than 5.96. The final pH of the solution was adjusted to between about 5.96 and 6.04.

To the buffered solution prepared as described above was added 18.5 mL of SIMPLE GREEN (Sunshine Makes Inc., Huntington Harbor, California). The pH of the resulting solution was adjusted by addition of concentrated hydrochloric acid or 4N potassium hydroxide as needed

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tissue enhancing composition

to obtain a pH of about 5.96 to 6.04. The resulting Tissue Enhancing Composition may be used to enhance the immunohistochemical staining of tissue samples.

Ex. #2. Method for the Preparation of Tissue Samples for Staining.

Formalin-fixed, Paraffin-embedded tissues were cut into 3 micron sections and placed on positively charged slides ploy-L-lysine slides. The sections were dried for 1 hour at a temperature of 58°C. The sections were then placed in a TISSUE-TEK staining dish (Miles Inc., Elkhart, IN, #4457) and sufficient Tissue Enhancing Composition, prepared as descried above, was added such that the tissue samples were substantially submerged within the solution. A second TISSUE-TEK staining dish was filled with a similar amount of Tissue Enhancing Composition. A pressure cooker (Presto Super Six Pressure Cooker #01263, National Presto Industries, Eau Claire, WI) was filled with tap water to a depth of approximately 1 inch. Both the TISSUE-TEK dish containing slides and Tissue Enhancing Composition, and the dish with only Tissue Enhancing Composition, were placed within the pressure cooker and the pressure cooker was sealed. The pressure cooker was heated on an electric burner until the temperature reached approximately 120°C at a pressure of about 2 atm. Once these conditions were reached the pressure cooker was heated for an additional 10 minutes. After this time the pressure cooker was removed from the electric burner and partially cooled. The pressure was vented and the TISSUE-TEK dishes were removed. The slides were then removed from the first TISSUE-TEK dish and transferred to the second TISSUE-TEK dish, which contained hot Tissue Enhancing Composition. An IHC wash buffer was prepared by dissolving a Phosphate Buffered Saline with Tween 20 packet (Sigma Chemical Co., St. Louis, MO #P-3563) in 1L of deionized water. The pH of the IHC wash buffer was adjusted to between 7.1 to 7.3 by addition of concentrated hydrochloric acid or 4N potassium hydroxide. After five to ten minutes the slides were

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removed and washed with an IHC wash buffer. IHC wash buffer may be obtained from Cell Marque Corp., Austin, Texas.

Exp. #3 Immunohistochemical Staining of the Tissue Samples.

The staining of tissue samples is detailed in the Cell Marque 1998 and 2000 Products and Reference Guide, which are incorporated by reference as if fully set forth herein. Tissue sections on slides (prepared as described above) were treated with a solution of excess antibody for an appropriate amount of time, such time varying depending on the antibody and the protocol being used. The slides were rinsed with an IHC wash buffer. The slides were placed in a Peroxide Block solution for 10 minutes. After this time the slides were rinsed with IHC wash buffer The slides were then placed in a Biotinylated Link solution for 10 minutes at room temperature. After this time the slides were rinsed with IHC wash buffer. The slides were then placed in a Label solution for 10 minutes at room temperature. The Label solution enhances the sensitivity of the antibodies toward the chromogen. After this time the slides were again washed in an IHC wash buffer. The slides were placed in Chromogen solution for 10 minutes at room temperature. After this time the slides were removed and rinsed with de-ionized water. The slides were then placed in hematoxylin Counterstain for 30 seconds at room temperature. The slides were finally rinsed with IHC wash buffer and following an appropriate mounting procedure a coverslip is placed over the tissue samples. All of the above mentioned solutions were obtained from Cell Marque Corp., Austin, Texas.

Exp. #4. Evaluation of the Staining Ability.

The formalin-fixed, Paraffin-embedded tonsil tissue samples were treated with two compositions in the manner described in Experiment 2. The first composition (Composition 1) was

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Hepatitis C, TORDJI-22	+++	+++++
HSV I & II, 045-A/1930-B	+	+++++
bCG, Polyclonal	+++++	+++++
Kappa, L1C1	+	+++++
Lambda, HP6054	+	+++++
Melanoma, HMB45	++++	+++++
Myleine Basic Protein, Polyclonal	+++++	+++++
Neurofilament, BF.10	++++	+++++
NSE, E27	++++	+++++
P53, D07	++++	+++++
PLAP, Polyclonal	+++	++++
PR, hPRa3	++++	+++++
PSAP, PASE/4LJ	+++	+++++
S-100, 4C4.9	++++	+++++
Vimentin, V9	+++++	+++++

A control sample was tested in which a formalin-fixed, tonsil tissue embedded in paraffin was heated in Composition 1 without any prior deparaffinization step. This tissue sample showed no staining in the presence of a variety of antibodies.

In addition to the results shown above, additional tests were performed using breast, prostate, thyroid, appendix, brain, lymph, skin, pancreas, colon, muscle bone marrow, and placenta. Staining with these tissue samples also showed that the use of Composition 2 improved staining of these embedded tissue samples without any prior removal of the embedding medium. It should be noted that the samples treated with Composition 1 were deparaffinized prior to treatment, but the samples treated with Composition 2 were not deparaffinized prior to treatment. The use of a composition

prepared according to the above embodiments, such as Composition 2, allows this deparaffinization step to be omitted and yet staining of the tissue samples of comparable or even better quality may be obtained.

While the above Exp. #4 was performed on tissue samples that have been heated in the above mentioned compositions, it should be appreciated that the reactions may also be performed at temperatures lower than described in the experiments. The treatment temperature may effect the rate of the tissue enhancing process. The rate of embedding medium removal, enhancement of the tissue sample, and rehydration of the tissue sample may all increase as the temperature is elevated. When the temperature is lowered, e.g. when the reaction is run at room temperature, the rate of these three processes may also be lowered. The lowering in rate may not have an effect on the enhancement of the tissue sample toward immunohistochemical staining, but may have an effect on the time period required for such enhancement to occur.

The use of a composition prepared according to the above embodiments may allow three steps to be performed in a substantially simultaneous manner: (i) removal of the embedding medium from the tissue; (ii) improvement of the immunohistochemical staining of the tissue in comparison to tissue that has not been contacted with the composition; and (iii) rehydration of the tissue sample. The importance of each of these three steps may be illustrated in Exp. #4. When a composition that only performs two of the three steps mentioned above is used, enhancement of the tissue samples may not be as pronounced as when all three steps are performed by a single composition. When Paraffin-embedded tissue samples are treated with a composition including only a tissue activating agent in water, little or no staining is seen. By removing the paraffin embedding medium prior to treating the tissue sample with a composition including a tissue activating agent in water, the staining

What is claimed is:

1. (once amended) A liquid composition for preparing tissue for immunohistochemical staining, wherein the tissue is substantially embedded in a tissue embedding medium, [comprising:] consisting essentially of

[a removing agent for the tissue embedding medium,] an emulsifier wherein the [removing agent] emulsifier is adapted to substantially remove the embedding medium from the tissue during use;

a tissue activating agent, wherein the tissue activating agent is adapted, when contacted with the tissue during use, to improve immunohistochemical staining of the tissue in comparison to tissue that has not been contacted with the tissue activating agent; and a

water in a sufficient quantity to substantially hydrate the tissue [during use];

[and] wherein, when the composition is contacted with tissue during use, the composition is [adapted to] capable of substantially remov~~e~~ing the embedding medium from the tissue, to improve immunohistochemical staining of the tissue in comparison to tissue that has not been contacted with the composition, and to substantially hydrate the tissue.

2. (once amended) A composition for enhancing [the] antigenicity of a fixed, embedded, slide-mounted tissue, the composition comprising:

a buffered solution of about .01 [miles] moles of trisodium citrate [dehydrate] dihydrate, and about 1000 milliliters of water;

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, the Simple Green comprising about 5.8% ethylene glycol mono-butyl ether, about 3.75% nonylphenol ethoxylate and about 1.5% tetrapotassium pyrophosphate; and

about 18.5 milliliters of SIMPLE GREEN®; and

an amount of [sufficient] reagent to adjust [the] pH to between about 6.0 and about 5.96.

(once amended) sufficient

3. An aqueous composition for enhancing [the] antigenicity of a fixed, embedded, slide-mounted tissue, the composition comprising: paraffin

a surfactant in a volume up to about 25%

[up to about 25% by volume of surfactants]

in a weight of up to 10%

[up to 10% by weight of] citric acid and alkaline citrate salts; and

sufficient [concentrated] acid to adjust [the] pH to acidity.

(once amended) for immunohistochemical staining

4. A method for using the composition of Claim 2, for preparing a fixed, embedded, paraffin dehydrated, slide mounted tissue for receipt of a stain thereon, including the steps of: immunohistochemical

contacting the slide with the composition and heating to a temperature of at least 80°C for a time sufficient so that the tissue becomes substantially hydrated;

removing the slide from the composition; and

washing the slide in an appropriate buffer solution to remove any remaining composition on the slide.

(once amended)

5. A method for using the composition of Claim 3 [for preparing a fixed, embedded, dehydrated, slide mounted tissue for receipt of a stain thereon,] including the steps of:

contacting the slide with the composition and heating to a temperature of at least 80°C for a time sufficient so that the tissue becomes substantially hydrated;

removing the slide from the composition; and

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washing the slide in an appropriate buffer solution to remove any remaining composition on the slide.

(once amended) receiving an immunohistochemical

6. A method for preparing a fixed, Paraffin-embedded, dehydrated, slide-mounted tissue, for [the receipt of a] stain thereon[:] comprising an emulsifier, a tissue activating agent and water, wherein the single liquid composition is

the method comprising the steps of: providing a first batch of a single liquid composition capable of substantially removing the paraffin, substantially unmasking the tissue, and substantially hydrating the tissue;

immersing the slide containing the tissue thereon in said single liquid composition for a combination of time and temperature sufficient to substantially remove the paraffin, substantially unmask the tissue, and to hydrate the tissue; and

rinsing the slide.

cancel 7. The method of claim 6 wherein the single liquid composition of the providing step includes an aqueous, emulsifying solution.

(once amended) 8. The method of claim [7 when] 6 wherein the aqueous emulsifying solution is a detergent and water.

(once amended) 9. The method of claim 8 wherein the detergent of the single liquid composition is in [the] a concentration range of between .1 and .5 percent.

(once amended) 10. The method of claim [7] 8 wherein the detergent of the single liquid composition is [at least one] one of the following: Igepal-630, Tween20, Brij 35, Brij 99, Triton X-100, CD TAB, [and] or Tween 80.

cancel 11. The method of claim 7 wherein the single liquid composition further includes an unmasking agent.

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12. (once amended) The method of claim [11] 6 wherein the [unmasking] tissue acting agent is one of the following: a chelator or a buffer.

13. (once amended) The method of claim 6 wherein the providing step includes the step of adjusting [the] pH of the single liquid solution to acidity.

14. (once amended) The method of claim 13 wherein the providing step includes the [sep] step of adjusting [the] pH to basicity.

15. (once amended) The method of claim 6 wherein the single liquid composition is [biodrgarduable] biodegradable and non-toxic.

16. The method of claim 6 wherein the providing step includes the providing of a second batch of the single liquid composition further including the step of reimmersing the slide containing the tissue in the second batch of the single liquid composition before the rinsing step.